

Remarks

Claims 2, 6, 12, 15, 16, 18 and 23 are under examination after the cancellation of claims 1, 3-5 and 8-11, and the addition of new claim 23. Claims 2, 6, 12, 15 and 20 are amended to better define the invention. Due to the restriction of the claims, claims 6, 12 and 20 are amended to recite only SEQ ID NO:6. No new matter is believed added by these amendments.

Rejection Under Obvious Type Double Patenting

The provisional rejection of claims 1-6, 12, 15, 16, 18 and 20 made in paragraph 16 of the Office Action mailed 08/27/03 under the judicially created doctrine of obviousness-type double patenting over claims 1, 2, 8 and 12-14 of the co-pending application SN 09/613,092, is maintained for reasons set forth therein.

The cancellation of claims 1 and 3-5 herein moots this rejection as to those claims. Furthermore, claims 1, 2, 8, and 12-14 have been cancelled in, and are no longer pending in, application number 09/613,092. Thus, the present rejection is not proper, and its withdrawal is respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph

The rejection of claim 2 made in paragraph 12 of the Office Action mailed 08/27/03 under 35 U.S.C. § 112, first paragraph, with regard to the deposit issue, is maintained for reasons set forth therein and here below. In this regard the present Office Action states the following:

Applicants cite MPEP 2164 and contend that the enablement requirement is met if one of skill in the art is enabled to make and use that which is defined by the claims. Applicants state that the standard for determining whether the specification meets the

requirement is: 'is the experimentation needed to practice the invention undue or unreasonable?' Applicants state that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. Applicants submit that the *Wands* factors are not limiting and can be used to determine if there is sufficient evidence to support a determination that a disclosure does not satisfy the requirement. Applicants assert that before any analysis of enablement can occur, the claims must be construed, and that the Office has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. Applicants submit that claim 2 is not directed to the listed monoclonal antibodies themselves, instead, to a peptide that immunospecifically binds to the listed monoclonal antibodies. Applicants state that the hybridomas and their respective monoclonal antibodies are disclosed in US patent 5,854,416 as indicated in the instant specification on page 12, which patent has been incorporated into the present application by incorporation. Applicants contend that antibodies can be prepared by many well known methods as indicated in pages 19 and 23, and Examples 1, 8 and 12 of the specification.

Applicants' arguments have been carefully considered, but are non-persuasive. Applicants are correct in that claim 2 is not drawn to a monoclonal antibody. However, in order to make the claimed peptide that is required to immunospecifically bind to the specific monoclonal antibody recited in claim 2, one has to have public access to the same monoclonal antibody. Despite the fact that methods of producing monoclonal antibodies are well known in the art, it would require undue experimentation for one of skill in the art to produce the exact same monoclonal antibody recited in the claim having the exact same binding characteristics or immunospecificity. Exact replication of the cell lines producing the recited monoclonal antibodies is an unpredictable event. Although respective monoclonal antibodies are disclosed in US patent 5,854,416, from the '416 patent it does not appear that hybridoma cell lines producing these monoclonal antibodies are publicly available except for the group to whom the '416 patent has been issued. There is no evidence of record that the hybridoma cell lines secreting the recited monoclonal antibodies may be reproducibly produced without undue experimentation, or that a suitable deposit has been made for patent purpose. A mere recitation of the various specific monoclonal antibodies is insufficient to meet the conditions under 37 C.F.R. 1.801-1.809.

A. Claim 2 has been amended to refer to an ATCC deposit of the monoclonal antibody 1B6E12H9, which is shown in Table 4 to bind SEQ ID NO:6. The deposit number has not yet been provided by ATCC, so it is not included in the claim. This claim and the specification will be amended to insert the deposit number and required depository information when the deposit number becomes available. Claim 2 is also amended to make it independent. Thus, there is no question whether applicants enable the mAb that defines the peptides being

claimed in amended claim 2. Having provided a specific antibody, it is a simple matter to test peptides of *Streptococcus pneumoniae* pneumococcal surface adhesion A protein (PsaA) against this antibody. Since this is what is claimed in claim 2, this rejection is believed to be overcome and its allowance is respectfully requested.

New claim 23 is directed to peptides that specifically bind the monoclonal antibody that binds the peptide of SEQ ID NO:6. Since the peptide and an antibody that binds it are provided, and because the generation of specific monoclonal antibodies against a peptide is routine, this claim is also enabled. Its allowance is, therefore, respectfully requested.

B. The rejection of claims 12, 15, 16, 18 and 20 made in paragraph 14 of the Office Action mailed 08/27/03 under 35 U.S.C § 112, first paragraph, as allegedly being non-enabled with regard to the scope, is maintained. In this regard the present Office Action states the following:

With regard to the peptide variants having 80% identity to SEQ ID NO: 6 or an immunogenic fragment of 80% identical peptide variants of SEQ ID NO: 6, Applicants contend that page 19 discusses using procedures to identify and produce an allelic immunogenic peptide. Applicants state that lines 9-20 on page 22 and Examples 4-7 and 14 discuss how to identify immunogenic fragments that elicit protective immunity and how to screen the identified peptides. Applicants submit that 'even if significant experimentation may be necessary, there is sufficient guidance within the specification to make the experimentation necessary to make and use the claimed peptides and compositions simply routine'.

Applicants' arguments have been carefully considered, but are non-persuasive. The peptide, the peptide variant with 80% identity, and/or immunogenic fragments thereof claimed in claims 12, 16, 18 and 20 are required to confer protective immunity against *S. pneumoniae* infection. The peptide variant or an immunogenic fragment thereof claimed in claim 15 is required to be at least *S. pneumoniae*-specific. As set forth previously, unpredictability is one of Wands factors for enablement. In the instant case, the claimed peptide variant having at least 80% sequence identity to the peptide of SEQ ID NO: 6 is required to be immunogenic, diagnostic and/or are protective against *S. pneumoniae* such that it is of use as a therapeutic, prophylactic or diagnostic composition. The claimed peptide variant is minimally required to be *S. pneumoniae*-specific. Although a microbial polypeptide or protein is expected in the art to generally induce specific antibodies, the ability of peptide variants having at least 80% sequence identity, i.e., as much as 20% non-identity, to the peptide of SEQ ID NO: 6, to confer protective immunity against a

microbial disease, pneumonia in the instant case, or to serve as a *S. pneumoniae*-specific diagnostic reagent, is not predictable.

Claim 12:

Claim 12 has not at any time recited any sequence variants of SEQ ID NO:6. Rather, claim 12 as filed recited a composition comprising SEQ ID NO:6 and fragments of SEQ ID NO:6. Thus, most of the present rejection does not apply to claim 12. If the Examiner believes that it does, she is respectfully requested to point to the language in the claim she believes conveys that idea. A fragment of a peptide (as contrasted with a variant) is nothing more than a subpart of the peptide, and this term does not by itself suggest any variation of the primary structure of the peptide. In other words, the relationship of the amino acids is the same with deletion of terminal peptides only. Combining this with the requirement that the peptide be protective means that the fragment must be at least 6 amino acids in length, because this is what is understood in the art as the minimum size of an epitope, and the peptide must comprise an epitope to be immunogenic or protective. Thus, the number of peptides that can be fragments of the 15 amino acid peptide of SEQ ID NO:6 is quite small. Thus, the quantity of work required to test such peptides would be within the scope of routine experimentation.

Nevertheless, applicants have amended claim 12 to delete reference to fragments. As such the claim recites a “therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsaA and that are immunogenic against *S. pneumoniae*, the peptides comprising amino acid residues whose amino acid sequences are chosen from the

group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and an immunostimulatory carrier, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.” As this claim does not possess the variability that is asserted in this rejection, the rejection of claim 12 should be withdrawn.

Claim 15:

Only claim 15, and claims 16 and 18 which depend therefrom, recite a peptide 80% similar to SEQ ID NO:6 or to immunogenic fragments of SEQ ID NO:6. A peptide 80% similar to SEQ ID NO:6 can only have at most 3 amino acids that differ from SEQ ID NO: 6. This is a small number of peptides that can be routinely made and tested. Furthermore, fragments of SEQ ID NO:6 can be no fewer than six amino acids in length in order to be immunogenic, because this is what is understood in the art as the minimum size of an epitope (see also specification, page 22, lines 5-7), and the peptide must comprise an epitope to be immunogenic or protective.

Nevertheless, applicants have amended claim 15 to delete reference to fragments. Thus, it is only the 15 amino acid peptide of SEQ ID NO:6 to which the sequence variability applies.

Furthermore, Applicants have amended claim 15 to recite “90% identical to the peptide of SEQ ID NO:6” instead of “80% identical.” Support for this amendment is in the specification at page 22, line 31 – page 23, line 1. The number of peptide variants of SEQ ID NO:6 with 90% similarity is only 300, a small number that can be routinely made and tested, given the amount of experimentation viewed as routine in this art.

Claim 15 does not require any protective effect. The peptides of claim 15 only have to be

immunogenic. Even though the immunogenicity of a peptide is not an *a priori* certainty, it is routine to make and test the 90% similar peptides for immunogenicity. Thus, given that 90% similarity allows at most only 1 amino acid to differ from SEQ ID NO:6, predictability of outcome is not required to practice the invention without undue experimentation. The amount and nature of the analysis are predictable, and the fact that the analysis will establish immunogenicity/non-immunogenicity of a given peptide is also predictable. Unpredictability is only one of the Wands factors, and there is no legal basis to give it more weight than the other factors in a Wands/Forman analysis.

The legal analysis of *In re Wands* is applicable to the present facts which are analogous to the facts underlying the decision of *In re Wands*. The method claims at issue in *Wands* involved the use of an antibody wherein the “antibody is a monoclonal high affinity IgM antibody having a binding affinity constant for . . . [the antigen] of at least 10^9M^{-1} .” *In re Wands*, 858 F.2d at 734. This claim covers any monoclonal antibody, not just a specific monoclonal antibody, and the PTO argued that the Applicants failed to enable all monoclonal antibodies. *Id.* Briefly, the skilled artisan generates monoclonal antibodies by injecting an antigen into a host animal causing an immune reaction, isolating spleen cells, some of which produce the antibodies that bind the antigen, fusing the spleen cells with a cancerous myeloma cell producing a hybridoma, and then screening individual hybridomas to isolate those that produce antibodies that bind the antigen. *Id.* at 733-734. The PTO supported its non-enablement position by pointing out that 1) not all hybridomas produce antibodies that bind antigen, 2) not all hybridomas that bind antigen will

bind with an affinity of 109M-1 , and 3) the Applicants own data indicated that a small percentage of hybridomas actually produced monoclonal antibodies which fell within the scope of the claims. Id. at 738-739. The court rejected these arguments by stating,

cell fusion [hybridoma technology] is a technique that is well known to those of skill in the monoclonal antibody art, . . . [t]here was a high level of skill in the art at the time when the application was filed, and all the methods needed to practice the invention were well known . . . [and] it seems unlikely that undue experimentation would be defined in terms of the number of hybridomas that were never screened, . . . [and since] Wands carried out his entire procedure three times , and was successful each time in making at least one antibody that satisfied all of the claim limitations . . . Wands evidence thus effectively rebuts the examiner's challenge to the enablement of their disclosure.

Id. at 740. Furthermore, the Wands court made clear that the amount of and type of experimentation considered undue fluctuates for each type of art. Id. The quantity of experimentation lacks relevance outside an assessment of what is "routine experimentation" in the art. Id. Thus, the huge amount of "experimentation" that the skilled artisan would have to perform to practice Wands' invention: immunizing an animal, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the hybridomas for the desired characteristics, knowing that many hybridomas would not produce functional antibodies and not knowing which hybridomas would produced claimed antibody, was not undue experimentation because it was routine experimentation in the art of monoclonal antibody production. Id.

The screening peptide fragments of SEQ ID NO:6 or peptides/fragments 90% similar to SEQ ID NO:6 for immunogenicity and specific binding against a known antibody is even more routine than the generation of monoclonal antibodies as in Wands. It involves only the application of routine binding assays using the known antibody and routine immunogenicity

assays. These are techniques that are well known to those of skill in the immunogenic peptide and monoclonal antibody art. The facts in this case call for the Office to directly consider whether the synthesis and testing of the peptides of claim 15 even rises to the level of “experimentation” as that word is understood by the skilled scientist in this field. This is all that is required to enable claim 15, since no evidence of protection is required for that claim. Even more favorable to applicants than the facts in Wands, the present facts suggest much less work would be required to do the necessary screening, based on the finite number of peptides (300) involved. Thus, by applying the proper legal test to claim 15, it would be correctly concluded that the claim is enabled.

Incidentally, applicants note that *S. pneumoniae* specificity is not recited in the claim 15. In fact, the peptides listed in claim 15 are not PsaA peptides, but were identified in phage display of random peptides. What is clear is that SEQ ID NO:6 immunospecifically binds a monoclonal antibody that doesn’t bind naturally occurring proteins other than PsaA. Thus it mimics an epitope specific for the PsaA protein of *S. pneumoniae*.

The Office Action further states the following in regard to this maintained rejection:

The instant specification fails to teach how to produce a peptide variant having at least 80% sequence identity to the peptide of SEQ ID NO: 6 such that it is capable of serving as a specific diagnostic or therapeutic/prophylactic composition and is capable of conferring protective immunity to *S. pneumoniae* infection in a human or non-human subject, or capable of diagnosing *S. pneumoniae* infections. The specification provides no guidance as to which specific amino acids must be retained and which may be varied within the peptide of SEQ ID NO: 6 without causing any detrimental effect to the claimed peptide that is meant to induce a protective immune response in a subject against *S. pneumoniae* infection or to diagnose a *S. pneumoniae* infection. There is no guidance in the instant specification with regard to which amino acid variations, i.e., insertions, deletions, additions and substitutions, in the peptide would result in a peptide variant of the recited

percent identity or an immunogenic fragment thereof that would retain the three-dimensional structure and the functional integrity or biological/immunogenic competence of the native protein or peptide, without rendering it non-functional. Except for a full length 37-kDa protein of *S. pneumoniae* that confers protective immunity against challenge with a wild-type *S. pneumoniae*, there appears to be no evidence within the instant specification, as originally filed, showing that the peptide of SEQ ID NO: 6, or an immunogenic fragment thereof, or a variant thereof having 80% sequence identity to the peptide of SEQ ID NO: 6 is capable of conferring protective immunity against *S. pneumoniae* or capable of detecting *S. pneumoniae* in a diagnostic assay. There appears to be not even a showing that the unmodified 15 amino acid-long peptide of SEQ ID NO: 6, let alone its immunogenic fragment or variant having at least 80% sequence identity, does indeed confer protective immunity against *S. pneumoniae* or detects *S. pneumoniae* infection via a diagnostic assay. A review of the specification suggests that the 'Results' section on page 31 and Example 4 of the specification describe the protective ability of the full length 37-kDa protein of *S. pneumoniae*. Table 4 shows that the peptide of SEQ ID NO: 6 is 1B6 mAb-specific. Example 14 shows that the peptide of SEQ ID NO: 6, when conjugated to KLH and mixed with an adjuvant, is immunogenic in mice. The protection experiments described in Examples 4 and 5 are limited to a showing that the whole 37-kDa protein of *S. pneumoniae* confers protection in mice against challenge with a wild-type *S. pneumoniae*. The specific monoclonal antibodies recited in the claims were generated using the 37-kDa protein, and not by using the claimed peptide. There is no showing that the peptide of SEQ ID NO: 6, an immunogenic fragment thereof, or a variant thereof having at least 20% dissimilarity to SEQ ID NO: 6, is protective against *S. pneumoniae*. The *S. pneumoniae*-specificity of an immunogenic fragment of the peptide of SEQ ID NO: 6, or a variant of the peptide of SEQ ID NO: 6, or an immunogenic fragment of the variant as recited, is not established. This is important because the art reflects unpredictability as to which amino acids in a specific protein can be varied, i.e., replaced or added, without adversely affecting the functional properties of that specific protein. While it is known in the art that variation in one or more amino acids is possible in a given protein, the exact position within its amino acid sequence where replacements or variations can be made, with a reasonable expectation of success of retaining the protein's functional integrity, is not certain. A random replacement affecting the epitopic amino acid positions that are critical, for example, to the three-dimensional conformational structure and specific binding property of the protein, would result in a polypeptide that may be non-functional (i.e., non-immunogenic) or not optimally immunogenic or protective as a vaccine candidate, because such positions tolerate no or little modifications. As set forth previously, Houghten et al. (New Approaches to Immunization, *Vaccines* 86, Cold Spring Harbor Laboratory, p. 21-25, 1986) teach the criticality of individual amino acid residues and their positions in peptide antigen-antibody interactions. Houghten et al. state (see page 24):

One could expect point mutations in the protein antigen to cause varying degrees of loss of protection, depending on the relative importance of the binding interaction of the altered residue. A protein having multiple

antigenic sites, multiple point mutations, or accumulated point mutations at key residues could create a new antigen that is precipitously or progressively unrecognizable by any of the antibodies in the polyclonal pool.

Thus, the art reflects that variations in critical residues at specific positions in an amino acid sequence could result in a polypeptide which may induce an antibody that may not recognize or bind to the native polypeptide of a microorganism. In the instant case, this is important because one of the purposes of the instant invention is to produce a peptide variant of *S. pneumoniae* or an immunogenic fragment thereof in its biologically active, immunogenic and/or protective form for inducing a protective immune response or diagnosing a *S. pneumoniae* infection. The instant disclosure lacks guidance on the precise position(s), nature and extent of amino acid replacements or variations that can be made in the claimed peptide in order to produce a variant with 80% identity to SEQ ID NO: 6 or an immunogenic 'fragment' thereof or, and with regard to whether it would serve as an effective immunogen capable of conferring protective immunity against *S. pneumoniae* infection in a human or a non-human subject, or as an effective *S. pneumoniae*-specific diagnostic reagent.

Regarding the application of this part of the rejection to claim 12, the issue of enablement seems to revolve around the question of the therapeutic effect of SEQ ID NO:6, since the claim does not recite variants of SEQ ID NO:6. Under a Wands analysis, the unpredictability of protection is fully balanced by the routine nature and small amount of research required to test SEQ ID NO:6 for protective effect. Applicants do not believe that the Office is asserting that the nature of this type of test is other than routine. This combined with the fact that only peptides that comprise SEQ ID NO:6 need to be assayed, contradicts any assertion that undue experimentation would be required to practice the invention of claim 12. To the extent that the Office maintains this rejection, it would appear that it is applying a per se requirement for efficacy data, a position which is not supported in the law.

Furthermore, peptide compositions comprising the peptide of SEQ ID NO:6 have been

shown to reduce nasopharyngeal (NP) carriage of *S. pneumoniae*. See Johnson et al. (Inhibition of Pneumococcal Carriage in Mice by Subcutaneous Immunization with Peptides from the Common Surface Protein of Pneumococcal Surface Adhesin A, J. Clin. Infec. Dis., 185:489-496, 2002), attached as Exhibit A with relevant parts underlined. As can be seen, this paper shows that immunization with composition P79 (which contains SEQ ID NO:6/P2) reduced NP carriage in mice. Thus, there is evidence in the literature that confirms the protective immunogenic effect of peptides comprising SEQ ID NO:6. The process of modifying a single amino acid in this 15-mer (i.e., to make the 90% identical peptide) and testing it for protective immunogenicity is well within the skill in the art as evidenced by the study done in Johnson et al. For this reason, as well as the above reasons, claim 12 is enabled.

Regarding the application of this part of the rejection to claim 15, it is noted again that claim 15 requires that the peptide be immunogenic and no more. Thus, any issue raised in the Office Action regarding protection is not relevant to claim 15.

The Office takes the position that the application does not provide guidance as to which specific amino acids must be retained and which may be varied within the peptide of SEQ ID NO: 6 without causing any detrimental effect to the claimed peptide. While this type of guidance would be useful, it is not the only guidance that can satisfy the enablement requirement. Rather, what is required is guidance as to the structure of the claimed peptides and a teaching of how to make them and test their activity. In this case there is a very high level of structural guidance provided for each and every peptide covered by claim 15. In fact, at least 90% of each claimed

molecule is structurally defined, i.e., 14 of 15 amino acids are identical to SEQ ID NO:6. With this high level of structural definition, the amount and nature of work needed to define the other less than 10% of the structure is quite small. It is this high level of structural guidance that allows one to routinely make and test the full scope of peptides for the immunogenicity called for in claim 15. The Office Action states that “[E]xample 14 shows that the peptide of SEQ ID NO: 6, when conjugated to KLH and mixed with an adjuvant, is immunogenic in mice.” Thus, the Office admits that the application teaches not only how to test peptides for immunogenicity, but also that the base peptide is immunogenic. The making of variants of a known peptide calls for nothing more than generating a list of variants and fragments and synthesizing them using techniques so well known as to require no specific disclosure in the application. Thus, all that is required is the simple process of testing a relatively small number (300) of peptide variants and fragments of SEQ ID NO:6 for immunogenicity. This meets the enablement requirement as set forth in the statute and applied in cases like *In re Wands* and *Ex parte Forman*.

The current Office Action appears to put undue stress on the issue of predictability. As noted above, predictability is one factor the Office must consider, but not in the absence of direct consideration of other *Forman* factors such as the nature and amount of work required and the level of skill in the art. It is easy to understand why the Office chooses to focus so much attention on the predictability factor, because it allows the Office to simply reject embodiments for which there is not direct evidence of efficacy (i.e., 100% predictability). However, in giving such disproportionate weight to predictability in this case, the Office is creating a requirement for

more data than the law actually requires. Despite the focus on predictability and data, the present case has an equivalent amount of data, and thus predictability, for the variants of claim 15 as Wands had for the monoclonal antibodies of the claims at issue in his case. The level of skill in the art is high, and those who work in this field expect to do the type and amount of work required to practice the invention of claim 15.

There is no factual basis provided in the Office Action as to why the work required to practice the invention would be considered undue by one in this field. Despite the statement in the Office Action that applicants arguments have been considered, the Office has ignored applicant's previous assertions regarding the routine nature and amount of work required to practice the invention. Applicants can understand why it is difficult for the Office to find any factual basis to conclude that this work more than routine, because of the frequency with which this type of work is done by those in the art. The routine nature of making and testing peptides for antigenicity and immunogenicity is found in monographs such as Barany G, Merrifield RB. In: Gross E, Meienhofer J, eds. The peptides. Vol I. New York: Academic Press, 1980:1-284. Furthermore, Tam JP. Multiple antigenic peptide system: a novel design of synthetic peptide vaccines and immunoassay. In: Tam JP, Kaiser ET, eds. Approaches to biological problems. New York: Alan R. Liss, 1989:3- 18, of record, teach routine protocols for making and testing antigenic/immunogenic peptides.

Nevertheless, the Office should provide a factual basis for disagreeing with applicant's characterization of this work as routine in amount and nature, or should accept it and apply it

appropriately as mitigation of unpredictability. In doing so, claim 15 should be found to be enabled.

Claims 16 & 18:

For claims 16 and 18, the issue of enablement relates directly to the enablement of claim 15 in the context of the scope of the claims. In the context of providing a credible basis for a protective effect, enablement of these claims has the same basis as the enablement of claim 12. Applicants have shown above how the scope of claims covering at least 90% similar variants are enabled due to the routine amount and nature of the work required to practice the full scope of the invention. This applies to claim 16 and 18, because they have this scope. Applicants have also shown above how the protective aspect of the claimed peptides is routinely established. Thus, for the same reasons claims 16 and 18 are enabled.

Furthermore, peptide compositions comprising the peptide of SEQ ID NO:6 have been shown to reduce nasopharyngeal (NP) carriage of *S. pneumoniae*. See Johnson et al. (Inhibition of Pneumococcal Carriage in Mice by Subcutaneous Immunization with Peptides from the Common Surface Protein of Pneumococcal Surface Adhesin A, J. Clin. Infect. Dis., 185:489-496, 2002), attached as Exhibit A with relevant parts underlined. As can be seen, this paper shows that immunization with composition P79 (which contains SEQ ID NO:6/P2) reduced NP carriage in mice. Thus, there is evidence in the literature that confirms the protective immunogenic effect of peptides comprising SEQ ID NO:6. The process of modifying a single amino acid in this 15-mer (i.e., to make the 90% identical peptide) and testing it for protective immunogenicity is

well within the skill in the art as evidenced by the study done in Johnson et al. For this reason, as well as the above reasons, claims 16 and 18 are enabled.

Claim 20:

Claim 20 is a therapeutic composition that comprises peptides defined essentially as in claim 12. Claim 20 recites “adjuvant” where claim 12 recites “immunostimulatory carrier.” As in claim 12, there is no recitation of sequence variants in claim 20. Thus, for all of the reasons claim 12 is enabled, so is claim 20.

The Office action concludes the rejection of claims 12, 15, 16, 18 and 20 as follows:

Therefore, undue experimentation would have been required to reproducibly practice the full scope of the invention as claimed currently, due to the lack of adequate and specific guidance, the lack of evidentiary support in the specification enabling a protective peptide of SEQ ID NO: 6, a protective or *S. pneumoniae-specific* variant peptide having at least 80% sequence identity to SEQ ID NO: 6 or an immunogenic fragment thereof, the nature of the invention, the state of the prior art, the quantity of experimentation necessary, and the art-demonstrated unpredictability in determining amino acid variations that are acceptable. *Ex parte Foreman*, 230 USPQ 546, 547 (*Bd. Pat. Appls. And Interf* 1986). One of skill in the art would not have been able to make the claimed product and use it, for example, for inducing anti-pneumococcal protective immune response in a subject, or as an *S. pneumoniae-specific* diagnostic reagent, without undue experimentation, because there is no disclosure as to what positions and what specific amino acid residues are varied or truncated. The claims are viewed as not meeting the scope of enablement provisions of 35 U.S.C § 112, first paragraph. The rejection stands.

After failing to directly address all of the relevant Forman factors, the Office concludes that claims 12, 15, 16, 18 and 20 fail the enablement test set forth in *Ex parte Forman*. Although reciting this factor as support for its conclusion, the Office Action does not substantively address the issue of the nature of the invention. In fact the nature of the invention involves simple immunochemistry and the application of well-known and regularly practiced assays. While mentioned in the conclusory sentence above, the Office Action does not substantively address the

state of the prior art. In fact, the prior art is replete with examples of the same nature and quantity of work as is required to practice the claimed invention, and these examples establish that this experimentation is considered routine. Although mentioned as a basis for the conclusion above, the Office Action does not substantively address the quantity of experimentation necessary to practice the invention. In fact, when compared to the huge amount of work permitted by the court in *In re Wands*, the quantity of work needed to make and use the peptides of the present invention is quite small. Failure of the Office to explicitly take these mitigating factors into account makes the analysis incomplete and unfairly biased against applicants. Because the Office has not properly conducted an analysis under either *In re Wands* or *Ex parte Forman*, the present rejection is improper, and its withdrawal is requested.

Rejection Under 35 U.S.C. § 102(e)

The provisional rejection of claims 1-5 and 8-11 made in paragraph 18 of the Office Action mailed 08/27/03 under 35 U.S.C § 102(e) as allegedly being anticipated by Sampson *et al.* (US 6,217,884), is maintained. In this regard, the Office Action states the following:

Applicants allege that the claims have been misread and misconstrued. Applicants submit that what is claimed is not a monoclonal antibody, the PsaA itself, or fragments of PsaA, but a composition comprising a purified peptide that immunospecifically binds to a monoclonal antibody obtained in response to immunizing an animal with PsaA. Applicants assert that the claimed peptide binds to the monoclonal antibody. Applicants acknowledge that Sampson *et al.* ('884) disclose an isolated nucleic acid of SEQ ID NO: 1 which encodes the 37 kDa PsaA of SEQ ID NO: 2 and a polypeptide encoded by the nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID NO: 1. Applicants acknowledge that columns 11-12 of Sampson *et al.* ('884) disclose fragments of the 37 kDa PsaA and that columns 12-13 disclose polyclonal and monoclonal antibodies which bind to the polypeptide encoded by a unique fragment of at least 10 nucleotides of SEQ ID NO: 1. Applicants agree that Sampson *et al.* ('884) disclose that an antibody can be raised to the PsaA fragment. Applicants readily admit that at lines 16-46 at column 13, Sampson *et al.* ('884) disclose a vaccine comprising an immunogenic

polypeptide encoded by a unique fragment of at least 10 nucleotides of SEQ ID NO: 1. Applicants allege that column 10 in the '884 patent has been misread. Applicants state that lines 16-67 of column 10 of the '884 patent describe a method of producing a 37 kDa pneumococcal surface adhesin protein by linking two peptides or polypeptides together by protein chemistry techniques. Applicants argue that these shorter and larger peptides and partial polypeptides 'may' or may not be immunogenic and that shorter sequences are joined to form the 37 kDa protein itself.

Applicants' arguments have been carefully considered, but are non-persuasive. As Applicants acknowledge, Sampson *et al.* ('884) disclosed PsA fragments, i.e., peptides, encoded by a unique at least 10 nucleotides of SEQ ID NO: 1, and larger peptides. The phrase 'at least 10 nucleotides of SEQ ID NO: 1' encompasses 20 nucleotides, 30 nucleotides, 40 nucleotides, 50 nucleotides, 60 nucleotides etc. As Applicants acknowledge, Sampson's monoclonal and polyclonal antibodies bind to such a polypeptide fragment or peptide. One of skill in the art would expect at least larger peptides or partial polypeptides from the 37 kDa protein to be immunogenic. One of skill in the art would reasonably expect at least one of Sampson's PsA fragments to bind immunospecifically to at least one of the monoclonal antibodies recited in claim 1. Thus, claims have not been misread and misconstrued. The disclosure of Sampson *et al.* ('884) anticipates the instant claims.

The cancellation of claims 1, 3-5 and 8-11 moots this rejection as to those claims.

Claim 2 recites a purified peptide that immunospecifically binds a specific monoclonal antibody not disclosed in the cited art. Monoclonal antibody 1B6E12H9 is not disclosed or suggested in the '884 patent. Thus, a novel subset of PsA peptides are defined by the claim. Withdrawal of this rejection and allowance of claim 2 is, therefore, respectfully requested.

Rejection Under 35 U.S.C. § 102(b)

The rejection of claims 1, 6 and 15 made in paragraph 19 of the Office Action mailed 08/27/03 under 35 U.S.C § 102(b) as allegedly being anticipated by Nuijens *et al.* (WO 9117258) as evidenced by Harlow *et al.* (*In: Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Chapter 5, p. 76, 1988), is maintained. The Office Action further states the

following:

Applicants acknowledge that Nuijens's Example II discloses a peptide with the amino acid sequence N-phe-ser-pro-val-ser-tyr-gln-his-asp-leu-ala-leu-C, which is a 12 amino acid peptide. Applicants assert that SEQ ID NO: 6 of the present invention is a 15 amino acid peptide: Arg-Ser-Tyr-Gln-His-Asp-Leu-Arg-Ala-Tyr-Gly-Phe-Trp-Arg-Leu. Applicants submit that although a portion of the sequences overlap, Nuijens's sequence does not anticipate the sequence of SEQ ID NO: 6. Applicants state that the fragment is an 'immunogenic fragment' and that the peptide of claim 15 is at least 80% identical to SEQ ID NO: 6, or at least 80% identical to an immunogenic fragment of SEQ ID NO: 6. Applicants allege that the attached sequence alignment shows a 40% query match of SEQ ID NO: 6. Applicants argue that Nuijens *et al.* do not disclose whether the 6 amino acid overlapping section of the peptide is immunogenic or immunogenic against *S. pneumoniae*. Applicants allege that the Office has not demonstrated whether the 6 amino acid sequence falls within the claims of the present invention.

Applicants' arguments have been carefully considered, but are non-persuasive. Contrary to Applicants' assertion, Nuijens' peptide is not a large sequence and it does not represent the entire human genome or the entire protein. Claim 1 does not place any size limit on the claimed peptide. Claim 1 fails to identify the claimed peptide by its structure, i.e., SEQ ID number. Therefore, the claimed peptide does not exclude Nuijens's SYQHDL-containing peptide. Since the recited peptide or protein is not identified by one or more structural limitations, it encompasses Nuijens's synthetic peptide. The instant claims contain a functional limitation without reciting any structure. Via the sequence identity with a six amino acid-long peptide from the SEQ ID NO: 6 of the instant specification, the Office has established that the prior art peptide has the exact identical structure of a peptide or fragment from SEQ ID NO: 6, SYQHDL, which is long enough to serve as an antigenic determinant or epitope. The source of this structurally identical peptide is irrelevant since a chemically synthesized peptide, not isolated *per se* from *Streptococcus pneumoniae*, is also encompassed within the scope of the claim. Thus, Applicants' argument that there is no basis for similarity of structure or functional or immunogenic correlation is inaccurate. The functional limitation, on which the prior art reference is silent, is considered as an intrinsic property of the prior art peptide, or a function of the prior art peptide uncharacterized at that time. With regard to Applicants' remark on the immunogenic nature of the peptide, it should be noted that SYQHDL does form an epitope for the PsaA-specific monoclonal antibody, 1B6, as evidenced by the teachings of Srivastava *et al.* (*Hybridoma* 19:23-31, 2000). The SYQHDL epitope-containing peptide existed at the time of the invention as taught by Nuijens *et al.* at line 13 on page 14 under Example II. Where the only difference between the claimed product and the prior art product is recited in the functional language, i.e., by what it does rather than what it is, it is incumbent upon Applicants, when challenged by the USPTO, to demonstrate that the prior art product does not actually possess those characteristics. Applicants have not shown that the underlying structure of the prior art peptide, SYQHDL, differs from that of the instantly recited peptide. The mere functional limitation does not impart a specific structure that distinguishes the peptide of the prior art from the

recited peptide. It should be noted that Nuijens *et al.* taught using the SYQHDL-containing peptide as an immunogen by conjugating to a protein carrier. Furthermore, Nuijens' SYQHDL qualifies as an immunogenic fragment of Applicants' SEQ ID NO: 6. The rejection stands.

The cancellation of claim 1 moots this rejection as to that claim.

Claim 6 is directed to SEQ ID NO:6 and immunogenic fragments thereof. An immunogenic fragment must be at least six amino acids in length, because this number of residues is generally viewed in the art of immunochemistry as the minimum length of an epitope (see also specification, page 22, lines 5-7, which recites this), and a peptide must contain an epitope to be immunogenic. To clarify this point, applicants have amended claim 6 to recite that the fragment "has at least six consecutive amino acids of SEQ ID NO:6" as supported in the specification as noted above. There is no six-amino acid region of SEQ ID NO:6 that is present in the peptide disclosed by Nuijens *et al.* In fact, what the Office Action asserts as the 6-amino acid region of identity (SYQHNL) is not even identical in that it contains asparagine (N) in the position where SEQ ID NO:6 recites aspartic acid (D). Contrary to what is stated in the Office Action, Nuijens *et al.* do not disclose SYQHDL. Thus, Nuijens *et al.* discloses, at most, a 4-amino acid region that is identical to a region of SEQ ID NO:6. This region is not immunogenic as required by claim 6, because it is not large enough to constitute an epitope. It also does not meet the recited size limitation of claim 6. Therefore, it does not anticipate claim 6.

Amended claim 15 recites peptides comprising variants of SEQ ID NO:6 with at least 90% similarity to SEQ ID NO:6. The amended claim also does not recite any fragments of SEQ

ID NO:6. The claimed peptide comprising the at least 90% similar variant of SEQ ID NO:6 comprises the full 15 amino acids of SEQ ID NO:6 with up to one amino acid variation in it within that 15-mer region. Since Nuijens et al. does not disclose any peptide that comprises a 15mer that has only one amino acid difference (i.e., at least 90% similarity) from SEQ ID NO:6, it has no disclosure that anticipates the peptides of claim 15.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending application are believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

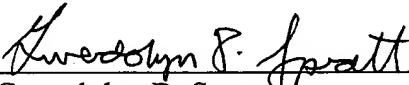


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Respectfully submitted,

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Gwendolyn D. Spratt

1-24-05
Date

Inhibition of Pneumococcal Carriage in Mice by Subcutaneous Immunization with Peptides from the Common Surface Protein Pneumococcal Surface Adhesin A

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Pneumococcal surface adhesin A (PsaA), a common protein expressed on all 90 pneumococcal serotypes, is a vaccine candidate. Three anti-PsaA monoclonal antibody phage display-expressed mono-peptides (15 mers), in various formulations as lipidated or nonlipidated multiantigenic peptides or as bi- or tripeptide constructs, were studied in a mouse nasopharyngeal carriage model to determine the inhibitory effect of induced antibodies on carriage of pneumococcal serotypes 2, 4, and 6B. Antibodies to each of the various peptides tested reduced carriage of the 3 serotypes. Reduction in carriage by nonlipidated multiantigenic peptide antibodies was highly variable (39%–94%; mean, 59%; standard deviation [SD], 20.2%); however, more-consistent results were observed in mice immunized with lipidated (56%–98%; mean, 69%; SD, 13.6%) and combination or bipeptide (55%–91%; mean, 76%; SD, 13.1%) formulations. These peptides are immunogenic, and their induced antibodies reduce carriage in mice. PsaA peptides demonstrate potential for being important new vaccines against pneumococcal carriage, otitis media, and invasive pneumococcal disease.

Streptococcus pneumoniae is a leading cause of morbidity and mortality and a major cause of bacterial pneumonia, meningitis, bacteremia, and otitis media in developed as well as developing countries [1–8]. The currently licensed vaccines available for immunoprophylactic control of these pneumococcal diseases are a first-generation 23-valent pneumococcal capsular polysaccharide (PS) vaccine and a second-generation 7-valent PS conjugate vaccine in which the PS is conjugated to the diphtheria protein CRM-197 [9]. In addition, several other investigational second-generation PS-protein conjugate vaccines, containing up to 11 serotypes, are presently in phase II and III trials in various parts of the world (The Gambia, Israel, Philippines, and South Africa) [10–14]. These pneumococcal vaccine formulations are based on the observation that antibodies to the PS protect against disease by enhancing phagocytosis [15].

The 23-valent PS vaccine covers ~90% of invasive pneumococcal disease in the United States, whereas the 7-valent conju-

gate vaccine covers ~80% of strains causing disease in children <5 years of age but only 55% of invasive pneumococcal disease among adults. However, the existence of ≥90 pneumococcal serotypes has complicated further development of these classes of vaccines. Although these vaccines cover the most prevalent serotypes found in the population at risk, they protect mainly against illness caused by those serotypes included in the vaccine. A limited amount of cross-protection among the serotypes has been observed with both the PS and the conjugate vaccine.

Alternate formulations of pneumococcal vaccines are under development. Considerable research is being conducted on potential third-generation common protein vaccines. Candidates under consideration are various pneumococcal cell-wall components and protein antigens, including pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A, and the protein PdB, a recombinant nontoxic pneumolysin [16–19]. These pneumococcal proteins, which are T cell dependent, are common to all pneumococcal serotypes and are likely to be highly immunogenic in humans and to elicit immunologic memory. In contrast to current pneumococcal vaccines, third-generation vaccine candidates will provide protection to a broader target population, from infants to elderly individuals, and to those at high risk. Furthermore, it is hoped that these will not be geographically specific and will provide protection against all pneumococcal serotypes.

PsaA, a 37-kDa lipoprotein expressed by all 90 *S. pneumoniae* serotypes, has been extensively characterized [16, 17, 19–23]. It has been shown to be immunogenic and protective against invasive pneumococcal disease and intranasal carriage in mice [17, 20–23]. Mutation analysis studies have demonstrated that changes in PsaA protein affect the binding of *S. pneumoniae*, indicating that PsaA plays a critical role in bacterial adherence and virulence [24]. In addition, research has indicated that PsaA

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determinants and epitopes are either completely or partially exposed on the surface of the cell [16]. These characteristics make PsaA a highly desirable candidate for use in the development of a third-generation pneumococcal vaccine.

However, use of proteins for vaccine development has limitations. Proteins are macromolecules composed of numerous amino acids that form 3-dimensional complexes. Because of the coiling and twisting of these complexes, potential immunogenic epitopes may be sterically hidden, obscured, or blocked and thus prevented from eliciting an immunologic response. In addition, proteins may have components that give rise to unwanted side effects or produce immunogens that hinder the wanted immunoreactive effect.

One way to avoid the disadvantages of using proteins for vaccines is to identify, isolate, and synthesize immunoreactive peptides (epitopes) of interest from the protein. Such epitopes are short, small amino acid units of the protein that can be linear and that are easier and less costly to synthesize for mass production. The use of synthetic peptides makes it possible to elicit an immunologic response to epitopes that remain cryptic during natural infection. In addition, it makes available antigenic and/or immunogenic peptides that may be difficult to prepare in quantity from native sources. Furthermore, the use of peptides rather than large, cumbersome, and costly proteins provides the opportunity to formulate vaccines with combinations of more immunoreactive units. Immunoreactive peptides, therefore, have the potential for being used to produce vaccines of the fourth generation.

Recently, we identified, isolated, sequenced, and described, with use of anti-PsaA mouse monoclonal antibodies, 3 idiotypes (mimotopes) from a phage display library reported elsewhere [25]. PsaA peptides corresponding to these idiotype sequences were shown to be 15 aa long and immunogenic in mice and demonstrated the ability to elicit an immunologic response that inhibited colonization of a pneumococcal serotype 2 isolate in a mouse nasopharyngeal (NP) carriage model [25]. Subsequently, we designed a study to determine whether peptides formulated from the sequence of these idiotypes would be viable candidates for the development of fourth-generation pneumococcal vaccines. This study, described herein, examined the inhibitory effects of antibodies induced by various peptide constructs formulated from these PsaA peptides on pneumococcal NP carriage in mice (i.e., ability of PsaA peptides to reduce the risk of acquiring pneumococci for carriage).

Materials and Methods

Bacterial isolates and growth conditions. Three isolates of *S. pneumoniae*, representing serotypes 2, 4, and 6B, were used for NP carriage experiments. Serotype 2 (isolate PLN-D39), a pneumolysin-negative mutant of D-39, was provided by James Paton (Women's and Children's Hospital, North Adelaide, Australia) [26]. Serotypes

4 (isolate DS2341-94) and 6B (isolate DS1756-94), which are typical strains, were provided by Richard Facklam (Centers for Disease Control and Prevention, Atlanta). All 3 isolates have been used frequently in animal model studies, in our laboratory and elsewhere. To ensure that multiple experiments could be initiated from the same lot of cells, a standardized stock culture of each pneumococcal isolate was prepared, as described elsewhere [27]. On initiation of an experiment, stock cells were cultured on blood agar (trypticase soy agar supplemented with 5% defibrinated sheep blood; BBL Microbiology Systems), transferred to brain-heart infusion broth (BBL Microbiology Systems) supplemented with 10% Levinthal's basal medium (BBL Microbiology Systems), and manipulated for animal inoculation, as described elsewhere [27].

Animals. Adult Swiss-Webster mice (ND-4; female; 8 weeks old) were obtained from Harlan Sprague Dawley. On arrival, mice were arranged in groups of 8 per cage. All animals were housed under standard conditions (25°C, relative humidity ~40%) with food and water available ad libitum. All mice were allowed to acclimate to their new environment for 1 week before experimentation.

Peptide synthesis. Three base peptides, designated "P1," "P2," and "P3," were used as templates to construct multiantigenic peptides (MAPs) and polypeptides (figures 1–4). The amino acid sequences of these peptides were defined from the 3 PsaA idiotypes mentioned above and have been described elsewhere (figure 1) [25]. In brief, the sequences for these base peptides were 15 aa in length. The motifs for these were found to align themselves along the PsaA protein strand at different locations and to have varying degrees of homology to the native protein. The P1 motif was aligned in region aa 132–146 and had a homology of 2 aa at positions aa 140 and 141. The alignments for the P2 and P3 motifs were found to be farther along on the protein strand at regions aa 206–220 and aa 250–275, respectively. The motif for P2 was found to have a 3-aa homology with the aligned region at positions aa 214, 215, and 216. Unlike the P1 and P2 motifs, the homology of the P3 motif consisted of 6 aa and was not continuous. Within the aligned region, the homologies occurred in 4 separate areas and were positioned at aa 253, 257, 260–261, and 266–267.

By using the sequences of the 3 base peptides as templates, three 4-arm, homologous MAPs in both the lipidated and nonlipidated forms were synthesized, along with nonlipidated bipeptide (4-arm, heterologous) and tripeptide (3-arm, heterologous) constructs (figures 2–4) and were studied in a mouse NP carriage model. The MAPs were synthesized on an ACT 396 multiple peptide synthesizer (Advanced ChemTech) by use of standard and modified Fmoc protocols, as described elsewhere [28–30]. The bi- and tripeptide

P1: T-V-S-R-V-P-W-T-A-W-A-F-H-G-Y

P2: R-S-Y-Q-H-D-L-R-A-Y-G-F-W-R-L

P3: L-V-R-R-F-V-H-R-R-P-H-V-E-S-Q

Figure 1. Amino acid sequences of 15-mer base peptides (P1, P2, and P3, described in Materials and Methods) used as templates to construct multiantigenic peptides and polypeptides [25]. A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

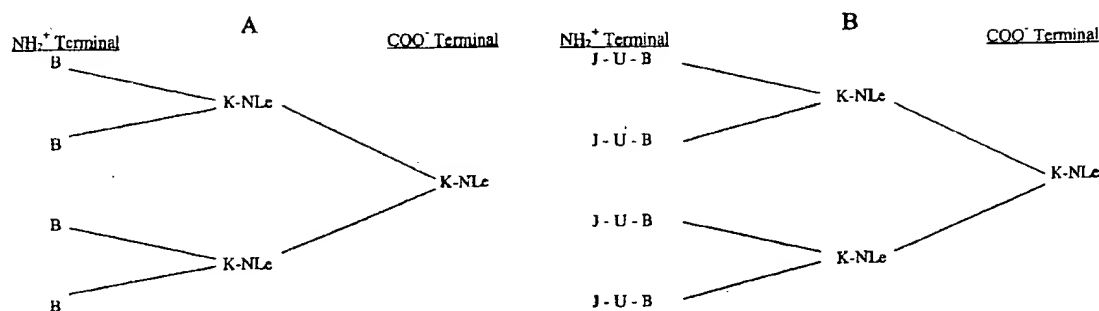


Figure 2. Basic structure of 6 multiantigenic peptides, 3 nonlipidated (A) and 3 lipidated (B). B represents three 15-aa base peptides (P1, P2, and P3, described in Materials and Methods) (figure 1) [25]. "J" represents the palmitoyl (palmitic acid) group, which is linked to the N-terminal of base peptides through a 3-aa chain linker (cysteine-serine-serine), designated "U" [31]. For P43 and P46, B = P1; for P44 and P47, B = P2; and for P45 and P48, B = P3. K-NLe, lysine-norleucine.

constructs were synthesized by the differential deprotection of Fmoc-Lys(Dde) or Fmoc-Lys(ivDde) (Novabiochem) with piperidine and hydrazine, using the method of Tam [31]. The crude peptides were analyzed for fidelity of synthesis by matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy, followed by 1 or more of the following procedures: amino acid analysis, high-performance liquid chromatography, capillary electrophoresis, or peptide sequencing. When necessary, the peptides were purified by semiprep high-performance liquid chromatography, using acetonitrile-water-trifluoroacetic acid buffer systems.

Structurally, the MAPs contain 4 branches that are homologous to each other and consist of P1, P2, or P3. Lipidated forms (designated "P43," "P44," and "P45") and nonlipidated forms (designated "P46," "P47," and "P48") are identical in structure, except that the former have a monosubstituted palmitoyl residue attached at the N-terminal of each base peptide unit by the 3-aa linker cysteine-serine-serine (figure 2). The MAPs were lipidated with palmitic acid to study the effect of an adjuvant on peptide immunogenicity and subsequent influence on NP carriage. P43 and P46 were previously referred to as peptides "43" and "46" [25]. Bipeptides, referred to as "P79" and "P80," are a combination of the base peptides (P1 and P2 or P1 and P3; figure 3). P79 contains 2 units each of P1 and P2, whereas for P80, P2 is replaced with P3. The tripeptide, identified as "P81" and consisting of 3 branches, is made up of 1 unit each of the base peptides (figure 4). In all cases, the peptide branches were synthesized on either a 3-arm or a 4-arm lysine-norleucine core (figures 2–4).

Mouse immunization and challenge. A mouse NP carriage model, described earlier, was used to determine the effect of immunogenic PsaA peptides on inhibition of NP pneumococcal carriage [32]. Mice were immunized according to a 3-dose regimen. Test mice ($n = 8$ for each peptide or peptide combination) received an initial dose of 100 μ g at day 0, followed by booster doses of 50 μ g of the appropriate peptide at 3 and 5 weeks after the initial dose. The lipidated MAPs were suspended in 100 μ L of sterile 0.01 M PBS (pH 7.2); all other peptides were mixed with 100 μ L of the adjuvant alum (alhydrogel-2%; catalog A1090B; Accurate Chemical and Scientific) at 6.3 mg/mL in PBS, to enhance the immunogenicity of the peptide. Control mice ($n = 8$) were similarly

immunized but without peptide. Each mouse was immunized subcutaneously between the shoulders. One week after the final booster immunization, all mice were challenged intranasally with 1×10^6 to 3×10^6 cfu of the appropriate *S. pneumoniae* isolate suspended in 10 μ L of 0.9% sodium chloride buffer (Abbott). Challenge inoculum was diluted serially 10-fold and was plated on blood agar plates to determine challenge dose.

Nasal wash and culturing. Five days after challenge, each mouse was euthanized, and the NP cavity was washed with sodium chloride buffer, as described by Wu et al. [33]. In brief, 100 μ L of wash was collected in a tube containing an equal volume of sodium chloride buffer (1:2 dilution) and then was diluted serially 3-fold to a final dilution of 1:486. Fifty microliters of each dilution was cultured on selective media (blood agar plates supplemented with 2.5 mg/L gentamicin to suppress growth of organisms other than *S. pneumoniae*). Cultures were incubated overnight at 37°C in an atmosphere of 5% CO₂ and high humidity. When possible, colonies were counted for each dilution, and the count was recorded. Carriage for a particular mouse was defined as the number of colony-forming units of challenge pneumococci in 50 μ L of collected nasal wash after counts for each dilution were adjusted to a nondi-

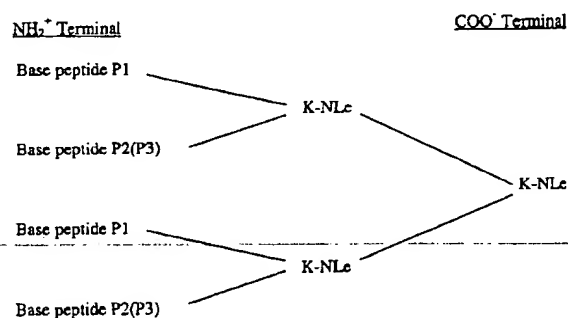


Figure 3. Basic structure of bipeptides P79 and P80. Base peptides (P1, P2, and P3, described in Materials and Methods) are linked together by the 2-aa linker lysine-norleucine (K-NLe) to form a 4-branched bipeptide (figure 1) [25, 31]. P79 consists of 2 units each of base peptides P1 and P2. In bipeptide P80, P2 units are replaced with P3 units.

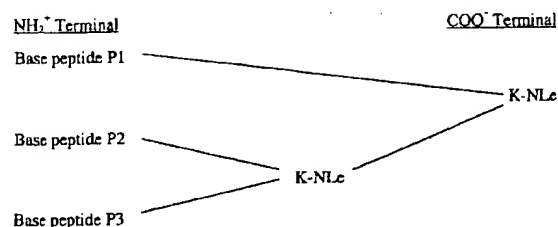


Figure 4. Basic structure of tripeptide P81. Base peptides (P1, P2, and P3, described in Materials and Methods) are linked together by the 2-aa linker lysine-norleucine (K-NLe) to form a 3-branched tripeptide (figure 1) [25, 31].

luted level and averaged. Sensitivity of carriage assay was 40 cfu/mL of nasal wash.

Statistical methods. Inhibition of pneumococcal carriage was determined by comparing the numbers of pneumococcal bacteria colonizing the NP area between immunized test mice and their paired immunogen-free control mice. Differences between groups were determined by the Mann-Whitney rank sum test, with level of significance set at $P < .05$. Statistical calculations were done with SigmaStat software (version 2.0; Jandel Scientific). Analysis was based on data generated from a single trial for each pneumococcal serotype and immunogen formulation.

Results

Effect of MAP immunization on inhibition of NP carriage.

In preliminary experiments, an immunologic response to MAP P43 was observed in mice (titers measured against native PsaA consistently $\geq 1:51,200$) when the animals were immunized according to the schedule described above (data not shown). On the basis of these results, pneumococcal carriage inhibition experiments were initiated on adult mice with the peptides described. When mice were immunized with nonlipidated MAP P46, P47, or P48 and challenged with *S. pneumoniae* serotype 2, 4, or 6B, reduction in NP carriage was observed for all and varied widely, from 39% to 94% (mean, 59%; SD, 20.2%), compared with the control group and as defined by the mean number of colony-forming units of challenge pneumococci colonizing the NP cavity (table 1). For those mice immunized with P46, reductions in NP colonization of 69% ($P = .04$) and 94% ($P < .01$) were observed for serotypes 2 and 4, respectively. For serotype 6B, the reduction was lower, 45%, but not significant ($P = .11$). Similarly, for P47-immunized mice, reduction in NP carriage ranged from 40% to 88%, with serotype 4 being inhibited the most. In addition, when mice were immunized with P48, the numbers of pneumococci colonizing the NP cavity were reduced by $\geq 39\%$ for all 3 serotypes. Similar but less variable results were seen when mice were immunized with lipidated MAP P43, P44, or P45 (table 2). Reductions in NP carriage were observed for all 3 serotypes and were $\geq 56\%$ (mean, 69%; SD, 13.6%). The largest decreases in carriage were again observed in mice challenged with serotype 4. Antibodies induced by these 3 lipidated MAPs in serotype 4-challenged

mice significantly ($P < .05$) reduced carriage by as much as 98% and no less than 74%. Lipidated MAP-immunized mice that were challenged with either serotype 2 or 6B experienced a reduction in pneumococcal carriage of 56%–65%.

Effect of immunization with MAP combinations or polypeptides on inhibition of NP carriage. The encouraging effects on inhibition of NP carriage produced by the immunologic response to the MAPs prompted further study of MAP combinations and polypeptide constructs. In all experiments in which mice were immunized with MAP combinations (P43 and P44 or P43, P44, and P45) or bipeptides (P79 or P80), inhibitions of NP carriage of $\geq 55\%$ (mean, 76%; SD, 13.1%; $P < .05$) were observed (table 3). Bipeptide-immunized mice challenged with any of the 3 serotypes experienced a reduction in NP carriage of $\geq 68\%$. Similar observations were seen in mice immunized with the tripeptide construct P81. These mice generated an immunologic response that reduced NP carriage of serotypes 2 and 4 by 53% and 82%, respectively ($P < .05$). Likewise, similarly immunized mice experienced a reduction in carriage of 49% ($P = .14$) when challenged with serotype 6B. Overall, an average decrease in pneumococcal carriage of 75% was observed in mice immunized with polypeptide constructs and challenged with serotype 2, 4, or 6B.

Discussion

The currently licensed pneumococcal vaccines, a 23-valent PS and a 7-valent PS-protein conjugate vaccine, along with several other polyvalent PS-protein conjugate vaccines currently undergoing trials, have serious limitations. The 23-valent vaccine is not licensed for children <2 years of age because of poor immune response. It induces protection in persons >65

Table 1. Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by nonlipidated pneumococcal surface adhesin A multiantigenic peptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide	Mean cfu (% reduction)	P
2		
P46	284 (69)	.04*
P47	1545 (40)	.24
P48	1344 (39)	.20
4		
P46	47 (94)	$<.01^*$
P47	46 (88)	$<.01^*$
P48	1438 (42)	.49
6B		
P46	1114 (45)	.11
P47	1312 (63)	.13
P48	1134 (58)	.03*

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ($n = 8$) recovered in 100 μ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with 10^6 cfu of *S. pneumoniae*.

*Significant difference vs. control mice injected with either PBS or alum ($P < .05$; Mann-Whitney rank sum test).

Table 2. Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by lipidated pneumococcal surface adhesin A multiantigenic peptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide	Mean cfu (% reduction)	P
2		
P43	2516 (56)	.13
P44	815 (64)	.11
P45	1766 (65)	.20
4		
P43	182 (74)	.048*
P44	23 (98)	<.01*
P45	142 (82)	<.01*
6B		
P43	1729 (63)	.02*
P44	1627 (58)	.01*
P45	544 (60)	.20

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ($n = 8$) recovered in 100 μ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with 10^6 cfu of *S. pneumoniae*.

*Significant difference vs. control mice injected with either PBS or alum ($P < .05$; Mann-Whitney rank sum test).

years of age that wanes significantly after 5 years [34–37]. Vaccine efficacy for the 23-valent vaccine is limited by the T cell-independent nature of the PS antigen and the number of serotypes represented in the vaccine [35]. Also, because antibodies to PS have limited cross-reaction with other serotypes, protection from this vaccine and from the various polyvalent PS-protein conjugate vaccines licensed or undergoing trials is afforded only to vaccine serotypes and selected other serotypes. Furthermore, the conjugate vaccine preparations have not proved to be more immunogenic than the conventional 23-valent vaccine in limited evaluations made to date among adults >50 years of age. Finally, the primary antibody response induced by PS-protein conjugate vaccines continues to manifest T cell-independent characteristics, such as dominance of IgG2 over IgG1 response in human adults [38–40]. On the other hand, PsaA is a component of the *S. pneumoniae* cell wall, which is highly conserved among the different serotypes of this organism and plays a key role in bacterial adherence and virulence. Antibodies against PsaA have been shown to be cross-reactive among all serotypes of *S. pneumoniae* [16]. As a protein antigen, it is capable of eliciting a T cell-dependent immune response. These properties of PsaA make it an ideal candidate for vaccine development.

Epitope mapping is a convenient procedure with which to delineate antigenic determinants of a molecule. In a previous study, we isolated, sequenced, and described 3 peptides from PsaA [25]. In the current study, we used various formulations of these peptides (MAPs, alone in the lipidated or nonlipidated forms, in combination with each other, or as bi- or tripeptide constructs) to determine the inhibitory effect of the immunostimulated antibodies on pneumococcal NP carriage in mice.

Our results indicated that inhibition of NP carriage among the pneumococcal isolates tested varied widely when mice were

immunized with nonlipidated PsaA MAPs. However, this variability decreased and was accompanied by higher rates of reduction in NP carriage when mice were immunized with lipidated MAPs, MAP combinations, or polypeptide constructs.

The ability of induced antibodies to our synthesized PsaA MAPs and polypeptide constructs to inhibit NP carriage of pneumococci can be based on several factors. These factors involve the peptide amino acid sequence, the peptide dosage used during immunization, and the adjuvant used to enhance peptide immunogenicity. The peptides used as templates in this study for synthesizing the MAPs were described earlier and were characterized as having a 2–6-aa homology to the native PsaA protein [25]. In addition, these homologous amino acids did not necessarily form a continuous motif. Sequences chosen from a phage display library are selected by monoclonal antibodies on the basis of their affinity to the given monoclonal antibody [41, 42]. Although these homologous amino acids are essential for binding to monoclonal antibody, it is possible that the intervening amino acids in the native protein could play a critical role in its immunogenicity. Also, amino acids distant in the primary sequence but adjacent in the tertiary structure of the protein may possibly play a critical role in modulating the immune response. Thus, use of a peptide sequence with greater homology to the native protein might be promising and might improve the immunogenicity.

In the present study, effects of dose ranging and scheduling on immunogenicity and NP carriage inhibition were not examined. However, studies of the immunogenicity of synthetic peptides indicate that immunogenicity is a function of peptide con-

Table 3. Inhibition of nasopharyngeal carriage of *Streptococcus pneumoniae* by pneumococcal surface adhesin A multiantigenic peptide combinations, bipeptides, or tripeptides in a mouse model.

<i>S. pneumoniae</i> serotype, peptide(s)	Mean cfu (% reduction)	P
2		
P43 + P44	603 (55)	.03*
P43 + P44 + P45	606 (60)	.04*
P79	1247 (78)	.03*
P80	718 (87)	<.01*
P81	580 (53)	.03*
4		
P79	212 (83)	.01*
P80	466 (82)	<.01*
P81	45 (82)	<.01*
6B		
P79	301 (91)	<.01*
P80	476 (68)	.02*
P81	872 (49)	.14

NOTE. Data are mean no. of colony-forming units (cfu) per mouse ($n = 8$) recovered in 100 μ L of wash from nasopharyngeal cavity. Mice were challenged intranasally with 10^6 cfu of *S. pneumoniae*.

*Significant difference vs. control mice injected with either PBS or alum ($P < .05$; Mann-Whitney rank sum test).

centration. A study of the peptide mimic of capsular polysaccharide of *Neisseria meningitidis* serogroup A (NmAPS) showed that the peptide is more immunogenic at lower concentrations. Optimal antibody response to NmAPS was observed in mice immunized with 1 and 5 μg of peptide, and the serum samples from the group immunized with 5 μg of peptide had the highest serum bactericidal assay titer [43]. In contrast, in a peptide mimic of capsular polysaccharide of *N. meningitidis* serogroup C (NmCPS), mice immunized with 50 μg of peptide had the best anti-NmCPS response [44]. Thus, the optimal dose depends on the peptide sequence in use and cannot be generalized across peptides. In certain cases, the individual MAP and tripeptide construct dose tested in this study against pneumococcal serotypes may not have been in the optimal range. However, when the combination MAPs or the bipeptide constructs were used, the dosage used appeared to be more optimal for reducing NP carriage. Overall, an obvious trend was observed among all mouse groups immunized with any of the tested peptides—a reduction in NP carriage, regardless of whether it was significant. To look at this in more detail, dose-ranging studies to optimize the effects of these peptides are currently under way.

The use of an appropriate adjuvant also could contribute toward the differences in a peptide response. In the current study, 2 methods were used to elicit an immunogenic response in mice. In the lipidated MAPs, a palmitoyl group was attached to the N-terminal end of the base peptides to enhance immunostimulation. Similarly, for the nonlipidated MAPs, bipeptides, and tripeptide, alum was used as the immunostimulator. Proteosomes have commonly been attached to peptides during peptide synthesis, to increase the immunologic response in animals [25]. The adjuvants palmitic acid, QS21, and alum have been used as immunostimulators in studies and can replace proteosomes as the adjuvant of choice to obtain an immune response [45–47]. Although both palmitic acid and alum were used in the current study to promote an immunologic response, there was no evidence to support one over the other as producing a greater response or better inhibition of NP colonization in the animal model. Although such a hypothesis was not examined during the present study, the problem of adjuvant choice is currently under investigation.

In the present study, PsaA was not used, and, therefore, no direct comparisons between our formulated PsaA peptides and the parent PsaA protein could be made with respect to their effect on NP carriage reduction. However, indirect comparisons can be made through 2 recent studies in which we participated [17, 21] that assessed NP carriage inhibition of *S. pneumoniae* by induced anti-PsaA antibodies. In one study, mice immunized with PsaA and challenged with serotype 6B experienced a 100-fold reduction in carriage, compared with controls [17]. Furthermore, these mice were observed to be colonized on average with ~ 140 cfu. In the current study, and for serotype 6B, the greatest carriage reduction was a 10-fold, or 91%, decrease and was observed in bipeptide P79-immunized mice. For the other peptide-

immunized mice, reduction in carriage averaged 45%–60%. However, for mice immunized with MAP P46, MAP combinations, and the tripeptide, the numbers of colony-forming units of serotype 6B colonizing the nasopharynx approached those observed in the previous study [17]. In the second study, NP carriage of serotype 2 averaged 30–70 cfu [21]. This was a 10-fold reduction over the control group. In our present study, and for peptide-immunized mice challenged with serotype 2, carriage reduction ranged between 39% and 87%. Furthermore, carriage rates in peptide-immunized mice were generally 10–100-fold higher (284–2516 cfu/mouse) than those observed in the PsaA-immunized mice of the previous study (28–66 cfu/mouse) [21]. In all 3 studies, mice were challenged intranasally with 10^6 cfu; however, for one study, the serotype 6B isolate used was not the same one we used in the present study. The ability of PsaA peptide-induced antibodies to inhibit NP pneumococcal carriage was not as robust in our study as was that observed in the 2 previous studies. The differences observed in reduction in carriage between the current and previous studies are not surprising but may have been magnified because of differences in experimental design (e.g., mouse strain, route of immunization, dosage, and adjuvant). In the previous studies, CBA inbred mice were used and were immunized intranasally with 150 or 500 ng of immunogen plus the adjuvant cholera B toxin [17, 21]. On the other hand, ND4 outbred mice were used in our current study and were immunized subcutaneously with 50 or 100 μg of immunogen. The adjuvant of choice in this case was either aluminum hydroxide (alum), for nonlipidated mono- and polypeptides, or palmitic acid, for lipidated peptides. Any of these differences, alone or in combination, may have influenced the final outcomes.

The focus of the present study was to examine the effect of various synthesized PsaA peptides on NP pneumococcal carriage in mice. The one common outcome in all cases when mice were immunized with one of the formulated peptides was the observation of reduction in pneumococcal carriage. Even though reduction in carriage was observed in every experiment, it was not always significant ($P < .05$). In some cases, this nonsignificance was most likely due to the sample size ($n = 8$) of the test group (i.e., lack of power). In other cases in which reduction in carriage was low and nonsignificant, increasing the immunization dosage might have enhanced this reduction and led to significant results. Therefore, the most important outcome of this study is not whether reductions in carriage were significant but, rather, that all formulated PsaA peptides were capable of eliciting an immunologic response in mice that, in turn, reduced pneumococcal carriage.

In conclusion, we studied 3 MAPs, either alone in the lipidated or nonlipidated form or in combination with each other, 2 bipeptides, and a tripeptide to determine the inhibitory effect of the induced antibodies on pneumococcal NP carriage in immunized mice. Overall, there was a noticeable inhibitory trend in the effect of immunostimulated antibodies induced to our various pep-

tides on NP carriage. Reduction in carriage was observed for all peptide formulations used to immunize mice. Inhibition of NP carriage was modest in mice immunized with the individual non-lipidated MAPs; however, when lipidated MAPs, MAP combinations, or bipeptides were used, variability was reduced and the ability of the immunostimulated antibodies to inhibit carriage was dramatically enhanced. These observations indicated that the anti-PsaA monoclonal antibody phage display-expressed peptides are immunogenic and reduce NP carriage in mice. These PsaA peptides demonstrate the potential for being important new vaccines against pneumococcal carriage, otitis media, and invasive pneumococcal disease, whether alone or in combination with other vaccine components.

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References

- Austrian R. The pneumococcus at the millennium: not down, not out. *J Infect Dis* 1999;179 (Suppl 2):S338–41.
- Dagan R, Englehard D, Peccard E, Israeli Pediatric Bacteremia and Meningitis Group. Epidemiology of invasive childhood pneumococcal infections in Israel. *JAMA* 1992;268:3328–32.
- Breiman RF, Spika JS, Navarro VJ, Darden PM, Darby CP. Pneumococcal bacteremia in Charleston County, South Carolina: a decade later. *Arch Intern Med* 1990;150:1401–5.
- Wenger JD, Hightower AW, Facklam RR, Gaventa S, Broome CV. Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. The Bacterial Meningitis Study Group. *J Infect Dis* 1990;162:1316–23.
- Istre GR, Tarpay M, Anderson M, Pryor A, Welch D. Invasive disease due to *Streptococcus pneumoniae* in an area with a high rate of relative penicillin resistance. *J Infect Dis* 1987;156:732–5.
- Giebink GS. Preventing pneumococcal diseases in children: recommendations for using pneumococcal vaccine. *Pediatr Infect Dis* 1985;4:343–8.
- Robbins JB, Austrian R, Lee CJ, et al. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J Infect Dis* 1983;148:136–59.
- Austrian R, Gold J. Pneumococcal bacteremia with specific reference to bacteremic pneumococcal pneumonia. *Ann Intern Med* 1964;60:759–76.
- Centers for Disease Control and Prevention. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 2000;49(RR-9):1–35.
- Wuorimaa T, Dagan R, Eskola J, et al. Tolerability and immunogenicity of an eleven-valent pneumococcal conjugate vaccine in healthy toddlers. *Pediatr Infect Dis J* 2001;20:272–7.
- Choo S, Finn A. New pneumococcal vaccines for children. *Arch Dis Child* 2001;84:289–94.
- Obaro SK, Adegbola RA, Chang IH, et al. Safety and immunogenicity of a nonavalent pneumococcal vaccine conjugated to CRM₁₉₇ and administered simultaneously but in a separate syringe with diphtheria, tetanus, and pertussis vaccines in Gambian infants. *Pediatr Infect Dis J* 2000;19:463–9.
- Mbelle N, Hubner RE, Wasas AD, Kimura A, Chang I, Klugman KP. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis* 1999;180:1171–6.
- Eskola J, Anttila M. Pneumococcal conjugate vaccines. *Pediatr Infect Dis J* 1999;18:543–51.
- MacLeod CM, Hodges RG, Heidelberger M, Bernhard WG. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* 1945;82:445–65.
- Russell H, Tharpe JA, Wells DE, White EH, Johnson JE. Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*. *J Clin Microbiol* 1990;28:2191–5.
- Briles DE, Ades E, Paton JC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* 2000;68:796–800.
- Alexander J, Berry AM, Paton JC, et al. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least 9 serotypes of *Streptococcus pneumoniae*. *Infect Immun* 1994;62:5683–8.
- Nabors GS, Braun PA, Herrmann DJ, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies. *Vaccine* 2000;18:1743–54.
- Crook J, Tharpe JA, Johnson SE, et al. Immunoreactivity of five monoclonal antibodies against the 37-kilodalton common cell wall protein (PsaA) of *Streptococcus pneumoniae*. *Clin Diagn Lab Immunol* 1998;5:205–10.
- De BK, Sampson JS, Ades EW, et al. Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*. *Vaccine* 2000;18:1811–21.
- Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 2000;68:3028–33.
- Talkington DF, Brown BG, Tharpe JA, Koenig A, Russell H. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb Pathog* 1996;21:17–22.
- Berry AM, Paton JC. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect Immun* 1996;64:5355–62.
- Srivastava N, Zeiler JL, Smithson SL, et al. Selection of an immunogenic and protective epitope of the PsaA protein of *Streptococcus pneumoniae* using a phage display library. *Hybridoma* 2000;19:23–31.
- Berry AM, Yother J, Briles DE, Hansman D, Paton JC. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. *Infect Immun* 1989;57:2037–42.
- Johnson SE, Rubin L, Romero-Steiner S, et al. Correlation of opsonophagocytosis and passive protection assays using human anticapsular antibodies in an infant mouse model of bacteremia for *Streptococcus pneumoniae*. *J Infect Dis* 1999;180:133–40.
- Barany G, Merrifield RB. In: Gross E, Meienhofer J, eds. The peptides. Vol 1. New York: Academic Press, 1980:1–284.
- Verheul AF, Udhayakumar V, Jue DL, Wohlhueter RM, Lal AA. Monopalmittic acid-peptide conjugates induce cytotoxic T cell responses against malarial epitopes: importance of spacer amino acids. *J Immunol Methods* 1995;182:219–26.
- Reed RC, Louis-Wileman V, Cosmai EV, et al. Multiple antigen constructs (MACs): induction of sterile immunity against sporozoite stage of rodent malaria parasites, *Plasmodium berghei* and *Plasmodium yoelii*. *Vaccine* 1997;15:482–8.
- Tam JP. Multiple antigenic peptide system: a novel design of synthetic peptide vaccines and immunoassay. In: Tam JP, Kaiser ET, eds. Approaches to biological problems. New York: Alan R. Liss, 1989:3–18.

32. Lipsitch M, Dykes JK, Johnson SE, et al. Competition among *Streptococcus pneumoniae* for intranasal colonization in a mouse model. *Vaccine* 2000; 18:2895-901.
33. Wu HY, Virolainen A, Mathews B, King J, Russell MW, Briles KE. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog* 1997; 23:127-37.
34. Douglas RM, Paton JC, Duncan LF, Hansman DJ. Antibody response to pneumococcal vaccination in children younger than five years of age. *J Infect Dis* 1983; 148:131-7.
35. van Dam JE, Fleer A, Snippe H. Immunogenicity and immunochemistry of *Streptococcus pneumoniae* capsular polysaccharides. *Antonie Van Leeuwenhoek* 1990; 58:1-47.
36. Rijkers GT, Mosier DE. Pneumococcal polysaccharides induce antibody formation by human B lymphocytes in vitro. *J Immunol* 1985; 135:1-4.
37. Douglas RM, Miles HB. Vaccination against *Streptococcus pneumoniae* in childhood: lack of demonstrable benefit in young Australian children. *J Infect Dis* 1984; 149:861-9.
38. Seppala I, Pelkonen J, Makela O. Isotypes of antibodies induced by plain dextran or a dextran-protein conjugate. *Eur J Immunol* 1985; 15:827-33.
39. Seppala I, Sarvas H, Makela O, Mattila P, Eskola J, Kayhty H. Human antibody responses to two conjugate vaccines of *Haemophilus influenzae* type B saccharides and diphtheria toxin. *Scand J Immunol* 1988; 28: 471-9.
40. Peeters CC, Tenbergen-Meekes AM, Poolman JT, Beurret M, Zegers BJ, Rijkers GT. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. *Infect Immun* 1991; 59:3504-10.
41. Bottger V, Stasiak PC, Harrison DL, Mellerick DM, Lane EB. Epitope mapping of monoclonal antibodies to keratin 19 using keratin fragments, synthetic peptides, and phage peptide libraries. *Eur J Biochem* 1995; 231: 475-85.
42. Hoess R, Brinkmann U, Handel T, Pastan I. Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3. *Gene* 1993; 128:43-9.
43. Grothaus MC, Srivastava N, Smithson SL, et al. Selection of an immunogenic peptide mimic of the capsular polysaccharide of *Neisseria meningitidis* serogroup A using a peptide display library. *Vaccine* 2000; 18: 1253-63.
44. Westerink MAJ, Giardina PC, Apricella MA, Kieber-Emmons T. Peptide mimicry of the meningococcal group C capsular polysaccharide. *Proc Natl Acad Sci USA* 1995; 92:4021-5.
45. da Fonseca DP, Joosten D, van der Zee R, et al. Identification of new cytotoxic T-cell epitopes on the 38-kilodalton lipoglycoprotein of *Mycobacterium tuberculosis* by using lipopeptides. *Infect Immun* 1998; 66: 3190-7.
46. Sheik NA, Rajananthanan P, Attard GS, Morrow WJ. Generation of antigen specific CD8⁺ cytotoxic T cells following immunization with soluble protein formulated with novel glycoside adjuvants. *Vaccine* 1999; 17: 2974-82.
47. Moore A, McCarthy L, Mills KH. The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. *Vaccine* 1999; 17: 2517-27.